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Multiple dependent claim(s) (if applicable)

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1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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29,768

REGISTRATION NUMBER

**CANDIDA ALBICANS TWO-COMPONENT HYBRID KINASE  
GENE, CaNik1, AND USE THEREOF**

**BACKGROUND OF THE INVENTION**

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Candida is an opportunistic yeast that lives in the mouth, throat, intestines, and genitourinary tract of most humans. In a healthy human body, the population of *Candida* is kept in check by the immune system and by a competitive balance with other microorganisms. But when the body's immune system is compromised, as in AIDS patients and in patients undergoing immunosuppressive therapy, *Candida* will grow uncontrolled, leading to systemic infection called "Candida mycosis." If left untreated, such systemic infections frequently lead to the death of the patients.

*Candida albicans* is a species of particular interest to scientists and doctors because 90% of all cases of *Candida* mycosis are caused by this species.

At present, the therapy principally available for invasive infections is based on relatively few antimycotics, such as amphotericin B and flucytosine, or the azole derivatives fluconazole and itraconazole. These antimycotics cause serious side effects, such as renal insufficiency, hypocalcaemia and anaemia, as well as unpleasant constitutional symptoms such as fever, shivering and low blood pressure. Amphotericin B is toxic to the kidneys, for example, and yet the pharmaceutical is therapeutic only if administered at dose levels near to being toxic. A discussion of the pharmaceuticals used for treatment and their corresponding side effects can be found, for example, in Boyd, et al., BASIC MEDICAL MICROBIOLOGY (2d ed.), Little, Brown and Company, (1981).

Given the deficiencies of conventional therapies against *Candida*, a need exists for developing pharmaceuticals that are effective in this regard and also safe to use. One step in the development of such pharmaceuticals requires a method for screening compounds in order to identify pharmaceutical candidates.

SUMMARY OF THE INVENTION

It therefore is an object of the present invention to provide an isolated polynucleotide sequence coding for a protein that is linked to phenotypic switching in *Candida albicans*.

It is a further object of the invention to provide a method for screening compounds to identify pharmaceutical candidates for effectively inhibiting the pathogenicity of *C. albicans*.

In accomplishing these and other objects, there has been provided, according to one aspect of the present invention, an isolated polynucleotide that codes for such a protein and that hybridizes, under stringent conditions, to the polynucleotide sequence of SEQ ID NO:1, shown below in Figure 1. In a preferred embodiment, the polynucleotide has the sequence of SEQ ID NO:3 (Figure 2). In another preferred embodiment, the protein displays a kinase activity.

In accordance with another aspect of the present invention, a method is provided for screening compounds to identify pharmaceutical candidates. The inventive method comprises the steps of (A) providing a plurality of cells from yeast species that exhibit phenotypic switching, at least some of which contain (i) a polynucleotide coding for a CaNIK1 protein and (ii) a promoter that is operably linked to the polynucleotide, such that the plurality of cells produces the protein; then (B) bringing the plurality into contact with a test substance; and (C) assessing what effect, if any, the test substance has on the expression of the DNA segment. Assessment step (C) can comprise, for example, of monitoring the level either of the protein or the corresponding mRNA transcript produced by the plurality of cells. In another embodiment, step (C) comprises monitoring the level of kinase activity, within the plurality, that typifies the protein.

In yet another embodiment of the present invention, a promoter is operably linked to a reporter gene. In this context, step (C) comprises monitoring the level of transcription of the reporter gene, after contact between the plurality of cells and the test substance.

Other objects, features and advantages of the present invention will become apparent from the following detailed

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description. It should be understood, however, that the detailed description and the specific examples, only indicate preferred embodiments of the invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the nucleotide sequence (top row) (SEQ ID NO:1) of the PCR product encoding the region spanning the H1 and D domains and the deduced amino acid sequence of the CaNIK1 protein (bottom row) (SEQ ID NO:2). The amino acid residues of functional domains are underlined. The three degenerate primers used to isolate the PCR products are shown as S1b1, S1b2, and S1b3.

FIGURE 2 shows the nucleotide sequence (top row) (SEQ ID NO:3) of the gene *CaNik1* and the deduced primary amino acid sequence of the CaNIK1 protein (bottom row) (SEQ ID NO:4). The beginning of each unique repeat is represented within the rectangle. The potential amino acid residues of different functional domains are underlined.

FIGURE 3 is a schematic representation of the anatomy of two alleles in two strains of *C. albicans* according to the present invention. All the functional domains are shown as white bold letters inside each rectangle. A few of the unique restriction enzyme sites are shown at the top of the rectangle. The start of the protein coding region is shown as ATG. WO-1 and CAI8 are the two strains analyzed in this invention. H1 and H2 are two identical alleles of the strain WO-1. H1-L and H2-S represent large and small alleles respectively in strain CAI8. The five hatched rectangular units in each allele represent repeat units described in this invention. The gray rectangular area encompassing *XhoI-PstI* in H2-S represents the region containing a deletion of approximately one repeat unit length.

FIGURE 4 illustrates the deletion strategy used to generate a homozygous deletion mutant, HH80, in strain CAI8. The region spanning *AflIII-XhoI* was deleted and substituted by a *hisG-UrabI* cassette in the plasmid pUNIK12.1 to create pCNH35

(Fig. 4c). Plasmid pUNIK12.1 (Fig. 4b) was derived by subcloning a PCR fragment using a pair of primers Slb8 and Slb7R and subcloning into pGEM-T easy plasmid vector.  $\lambda$ SA15.1 represent the lambda clone identified in a screen that contain the genomic fragment encompassing the entire *CaNik1* gene and the flanking DNA sequence.

FIGURE 5 shows the deletion strategy used to generate the homozygous deletion mutant in Red 3/6, an *ade2<sup>-</sup>* derivative of strain WO-1. The deletion cassette pABX12 (Fig. 5b) was generated by deletion of all the functional domains except H2 and substitution with the *ADE2* gene as an auxotrophic marker in pUNIK12.1 (Fig. 5c). Figure 4 provides a description of  $\lambda$ SA15.1.

Table 1 summarizes the effects of the *CaNik1* deletion in HH80 on growth in a variety of solution and conditions, high frequency phenotypic switching, and dimorphism.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

*Candida albicans* is capable of differentiating in a reversible fashion between a bud and a hyphal growth form. Each strain of *C. albicans* can also undergo high frequency phenotypic switching between a limited number of general phenotypes that differ in a variety of traits including putative virulence factors. The frequencies of both of these developmental programs are influenced by environmental conditions. For example, pH and temperature influence the transition between bud and hypha while temperature, UV, white blood cell metabolites and colony aging affect the frequency of high frequency phenotypic switching. The morphological changes made by *C. albicans* in response to environmental cues indicates that the organism uses a sensory mechanism to register and assess environmental alterations.

Autophosphorylating histidine kinases, also known as "two-component response regulators," have been found, in lower eukaryotes such as fungi and slime molds, to play a pivotal role in relaying various environmental signals into the cell for inducing appropriate responses and in providing these organisms

with the capacity to respond rapidly to an environmental perturbation. Two-component signal transducers all contain a sensory kinase, which autophosphorylates a histidine residue in response to an environmental cue, and a response regulator, which then is phosphorylated and, through a resultant conformational change, effects a signal that is transduced either directly to a molecular complex, as in the case of the bacterial *CheY* and the flagellar motor, or down a signal transduction pathway, as in the case of *SLN1*. These proteins have been shown to be involved in regulating morphogenesis and development in various prokaryotes and eukaryotes.

That two-component response regulators have been identified in other yeast species suggests that the two-component response regulators may also play a role in the developmental programs of *C. albicans*. The present invention relates to such a two-component response regulator, the hybrid kinase *CaNIK1* from *Candida albicans*. A link between the gene encoding *CaNIK1* and the processes of phenotypic switching that includes the differential expression of pathogenic genes is evidenced by work with a *CaNIK1*-deletion strain of *C. albicans*. See examples 3 and 5. Thus, *CaNIK1* is known to be involved in phenotypic switching.

Phenotypic switching is thought to be linked to the virulent characteristics of yeast. *Candida albicans* switches phenotypes with regard to its environment in order to maximize pathogenesis according to the demands of the particular environment. For example, in the WO-1 strain of *Candida albicans*, studies have shown that the yeast is more virulent in its opaque phenotype when located on the skin. When WO-1 is in the white phenotype, however, it is more pathogenic in systemic infections. A description of the relationship between the phenotypic switching and the pathogenic characteristics of *Candida albicans* can be found in Soll, "Switching and Gene Regulation in *Candida albicans*," in SOCIETY FOR GENERAL MICROBIOLOGY SYMPOSIUM 50 (1992). This relationship between phenotypic switching and pathogenicity can be exploited effectively, in a bioassay, for the purpose of discovering pharmaceutical candidates against *Candida albicans*.

## 1. Definitions

In this description, "**isolated DNA**" is a fragment of DNA that is not integrated in the genomic DNA of an organism. For example, the *CaNik1* gene is a DNA fragment that has been isolated from the genomic DNA of *C. albicans*.

As used herein, "**protein**" refers to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The essential nature of such analogues of naturally occurring amino acids is that, when incorporated into a protein, that protein is specifically reactive to antibodies elicited to the same protein but consisting entirely of naturally occurring amino acids. The terms "polypeptide", "peptide" and "protein" are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation. Exemplary modifications are described in most basic texts, such as PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES (2d ed.), T. E. Creighton, W. H. Freeman and Company, New York (1993).

As used herein, "**selectively hybridizes**" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, preferably 90% sequence identity, and most preferably 100% sequence identity (i.e., complementary) with each other.

The terms "**stringent conditions**" or stringent hybridization conditions includes reference to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions,



target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the  $T_m$  can be approximated from the equation of Meinkoth & Wahl, *Anal. Biochem.* 138: 267-84 (1984):  $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$ ; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe.  $T_m$  is reduced by about  $1^\circ\text{C}$  for each 1% of mismatching; thus,  $T_m$ , hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with  $\geq 90\%$  identity are sought, the  $T_m$  can be decreased  $10^\circ\text{C}$ . Generally, stringent conditions are selected to be about  $5^\circ\text{C}$  lower than the thermal melting point ( $T_m$ ) for the specific sequence and its complement at a defined ionic strength and pH. But severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or  $4^\circ\text{C}$  lower than the thermal melting point ( $T_m$ ); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or  $10^\circ\text{C}$  lower than the thermal melting point ( $T_m$ ); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or  $20^\circ\text{C}$  lower than the thermal melting point ( $T_m$ ). Using the equation, hybridization and wash compositions, and desired  $T_m$ , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired

degree of mismatching results in a  $T_m$  of less than 45°C (aqueous solution) or 32°C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY --HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays," Elsevier, New York (1993); and in Chapter 2 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing and Wiley-Interscience, New York (1995) (hereafter "Ausubel et al.").

Two nucleic acid molecules are considered to have a "substantial sequence similarity" if their nucleotide sequences share a similarity of at least 50%. Sequence similarity determinations can be performed, for example, using the FASTA program (Genetics Computer Group; Madison, WI). Alternatively, sequence similarity determinations can be performed using BLASTP (Basic Local Alignment Search Tool) of the Experimental GENIFO(R) BLAST Network Service. See Altschul et al., "Sequence Similarity Searches, Multiple Sequence Alignments, and Molecular Tree Building," in METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY, Glick et al. (eds.), pages 251-267 (CRC Press, 1993).

As used herein "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. Tissue-specific, tissue-preferred, cell type-specific, and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is one that is active under most environmental conditions.

As used herein "operably linked" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary

to join two protein coding regions, contiguous and in the same reading frame.

As used herein, "**expression**" refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

As used herein, "**expression vector**" is a polynucleotide molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements, including constitutive or inducible promoters, tissue-specific regulatory elements, and enhancers. Such a gene is said to be "operably linked" to the regulatory elements.

## 2. Isolating a *Candida albicans* Polynucleotide Segment Encoding CaNik1 Protein

An endogenous polynucleotide sequence from *Candida albicans* which encodes for the CaNIK1 protein was isolated using a polynucleotide probe derived from PCR amplification. See Example 1. Hybridization of the probe against a genomic library resulted in the determination of the full length polynucleotide sequence encoding the CaNIK1 protein. See Example 2. The full polynucleotide sequence encapsulating the CaNik1 gene is provided in Figure 2.

## 3. Nucleic Acids

The present invention provides, *inter alia*, isolated nucleic acids of RNA, DNA, and analogs and/or chimeras thereof, comprising a polynucleotide encoding a CaNIK1 protein or a polynucleotide probe which hybridizes to a polynucleotide encoding CaNIK1 protein. In this regard, the invention provides the nucleotide sequences of Figures 1 and 2. In addition, the present invention also provides other sequences as described below.

a. *Polynucleotides Encoding A CaNIK1 Polypeptide or Conservatively Modified or Polymorphic Variants Thereof*

As indicated above, the present invention provides isolated heterologous nucleic acids comprising a polynucleotide, wherein the polynucleotide encodes a CaNIK1 protein, disclosed herein in Figure 2, or conservatively modified or polymorphic variants thereof. Those of skill in the art will recognize that the degeneracy of the genetic code allows for a plurality of polynucleotides to encode for the identical amino acid sequence. Such "silent variations" can be used, for example, to selectively hybridize and detect allelic variants of polynucleotides of the present invention. Accordingly, the present invention includes polynucleotides that are silent variations of the polynucleotides of Figure 2. The present invention further provides isolated nucleic acids comprising polynucleotides encoding conservatively modified variants of CaNIK1 encoded by the sequences in Figure 2. Conservatively modified variants can be used to generate or select antibodies immunoreactive to the non-variant polypeptide. Additionally, the present invention further provides isolated nucleic acids comprising polynucleotides encoding one or more polymorphic (allelic) variants of polypeptides/polynucleotides.

b. *Polynucleotides That Selectively Hybridize*

The present invention also provides isolated nucleic acids comprising polynucleotides, wherein the polynucleotides selectively hybridize, under selective hybridization conditions, to a polynucleotide as discussed above. In this regard, the present invention encompasses polynucleotides that selectively hybridize, under selective conditions, to a polynucleotide as discussed above, excluding the polynucleotide of Figure 2. Thus, the polynucleotides of this embodiment can be used for isolating, detecting, and/or quantifying nucleic acids comprising the polynucleotides described above. For example, polynucleotides of the present invention can be used to identify, isolate, or amplify partial or full-length clones in a deposited library. In some embodiments, the polynucleotides are genomic or cDNA sequences isolated, or otherwise complementary to, a cDNA from a nucleic acid library. Preferably, the cDNA library comprises at

least 80% full-length sequences, preferably at least 85% or 90% full-length sequences, and more preferably at least 95% full-length sequences. The cDNA libraries can be normalized to increase the representation of rare sequences. Low stringency hybridization conditions are typically, but not exclusively, employed with sequences having a reduced sequence identity relative to complementary sequences. Moderate and high stringency conditions can optionally be employed for sequences of greater identity. Low stringency conditions allow selective hybridization of sequences having about 70% sequence identity and can be employed to identify orthologous or paralogous sequences.

*c. Polynucleotides Having at Least 60% Sequence Identity*

The present invention further provides isolated nucleic acids comprising polynucleotides, wherein the polynucleotides have a specified identity at the nucleotide level to a polynucleotide as disclosed above. In this regard, the present invention encompasses polynucleotides that have a specified identity to the polynucleotides discussed above, but are not the same as the sequence of Figure 2. The percentage of identity to a reference sequence is at least 60% and, rounded upwards to the nearest integer, can be expressed as an integer selected from the group of integers consisting of from 60 to 99. Thus, for example, the percentage of identity to a reference sequence can be at least 70%, 75%, 80%, 85%, 90%, or 95%.

4. Vectors

According to the present invention, the polynucleotide sequence encoding the CaNIK1 protein may be inserted into any suitable yeast vector with any method known to a person who has skill in the art. The vector will typically be comprised of a polynucleotide encoding the CaNIK1 protein operably linked to any suitable promoter which will direct the transcription of the polynucleotide in the intended host cell. Examples of suitable promoters include EF1 $\alpha$ 2 which is a constitutive promoter and is characterized in Sundstrom et al., *General Bacteriology*, 172: 2036-2045 (1990), and PCK1 which is an inducible promoter and is characterized in Leuker et al., *Gene*

192: 235-240 (1997). According to the present invention, the promoter is operably linked to the polynucleotide encoding for the CaNIK1 protein and inserted into a yeast transformation vector.

5 Yeast vectors are grouped into five general classes according to their mode of replication in the yeast: YIp, YRp, YCp, YEp, YLp. Comprehensive laboratory techniques regarding insertion of polynucleotides into yeast vectors can be found in Chapter 13 of Ausubel et al.

## 10 5. Bioassay

Another aspect of the invention is a bioassay useful for screening pharmaceutical candidates which can inhibit pathogenicity in *Candida albicans*. The bioassay is based on assessing a candidate's ability to inhibit expression or functionality of the *CaNik1* gene or its gene product, which as explained above, is linked to the virulent characteristics of the yeast. A bioassay according to the present invention comprises the following steps: transformation of cells from yeast species that exhibit phenotypic switching with a polynucleotide encoding CaNIK1 protein, and a promoter linked to the polynucleotide segment which can drive protein expression; effecting contact between the yeast cells and a pharmaceutical candidate; and analyzing the effect of the pharmaceutical candidate on inhibition of the expression of the *CaNik1* gene. In one embodiment, *C. albicans* cells harboring a *CaNik1* deletion are transformed with a suitable construct containing a CaNIK1-encoding polynucleotide, and an operably linked promoter.

## 30 A. Transformation of Yeast Cells

The present invention contemplates the use of yeast cells with a phenotypic switching pathway similar to that of *Candida albicans*. Srikantha et al., J. Bacteriol. 179: 3837-3844 (1997). Transformation of the cells can be accomplished through any means known to a person with skill in the art. One example of a yeast transformation procedure is the lithium acetate procedure whereby yeast cells are briefly incubated in

buffered lithium acetate and transforming DNA is introduced with carrier DNA. Addition of polyethylene glycol (PEG) and a heat shock trigger DNA uptake. An alternate method of transforming yeast cells is the electroporation procedure whereby concentrated cells are transformed using an exponential electric pulse. Comprehensive laboratory techniques regarding yeast transformation procedures can be found in Chapter 13 of Ausubel et al.

B. Contact of a Test Substance with Transformed Cells

According to the present invention, a test substance should make contact with at least some of a plurality of cells transformed with a polypeptide encoding *CaNik1*. Contact includes any exposure of the test substance to any surface of a transformed cell. A preferred method of contact would be incubation of the cells with the test substance.

The test substance includes any compound which may have characteristics inhibitory to the growth or the pathogenicity of *Candida albicans*. An example of a test substance is a pharmaceutical compound with antimycotic properties.

6. Assessing of the Effect of the Test Substance on *CaNik1* Gene Expression

According to the present invention, the effect of the pharmaceutical compound on *CaNik1* expression is analyzed after contact between the pharmaceutical compound and the plurality of transformed cells. *CaNik1* expression can be measured through any means known by a person with skill in the art. Examples of methods which monitor the level of gene expression are: measuring levels of *CaNIK1* protein and mRNA produced by the cells; or measuring the kinase activity within the cell; or monitoring the level of transcription of a reporter gene operably linked to a promoter.

An example of monitoring *CaNik1* expression is the measurement of levels of *CaNIK1* protein produced by the plurality of cells. This can be measured by performing two-dimensional gel electrophoresis using the techniques of isoelectric-focusing and SDS-polyacrylamide gel electrophoresis

followed by autoradiography of the gel. Comprehensive laboratory techniques regarding two-dimensional gel electrophoresis and autoradiography can be found in Chapter 10 and Appendix 3 of Ausubel et al.

5 Another example of monitoring *CaNik1* expression is to measure the level of mRNA encoded within the cell and produced by the plurality. mRNA levels within the cell can be measured with the following three techniques: Northern Blot, primer extension and ribonuclease protection. The Northern Blot  
10 procedure consists of fractioning mRNA with gel electrophoresis, transferring the mRNA fragments from the gel onto a filter and hybridizing the target mRNA molecules used a labeled DNA or RNA probe. The primer extension procedure includes hybridizing an oligonucleotide primer to the 5' end of the target mRNA and extending the primer using reverse transcriptase and unlabeled deoxynucleotides to form a single-stranded DNA complementary to the template RNA. The resultant DNA is analyzed on the sequencing gel. The yield of the primer extension product quantifies the amount of mRNA produced by the cell. The ribonuclease protection assay measures mRNA levels by hybridizing sequence specific RNA probes to sample RNAs. The probe anneals to homologous sequences in the sample RNA. The presence of target RNA is analyzed and quantified by gel electrophoresis. Comprehensive laboratory techniques regarding  
25 Northern Blot, primer extension and ribonuclease protection assays can be found in Chapter 4 of Ausubel et al.

A third example of monitoring *CaNik1* expression is to monitor the level of kinase activity within the plurality of cells. Kinase activity within the cells can be monitored by  
30 labeling ATP with  $^{32}\text{P}$  in vitro. The labeled ATP acts as the donor substrate, and the *CaNIK1* protein acts as the acceptor substrate. Phosphotransfer is detected as the accumulation of  $^{32}\text{P}$ -labeled protein within the cell. The accumulation of protein is measured with polyacrylamide gel electrophoresis and autoradiography. Target kinase activity can be  
35 distinguished from background kinase activity with autophosphorylation of the *CaNIK1* protein on polyacrylamide gel. Comprehensive laboratory techniques regarding



phosphorylation and measurement of kinase activity can be found in Chapter 18 of Ausubel et al.

In a further example, a reporter gene is operably linked to a promoter and the level of transcription of the reporter gene is monitored after contact between the plurality and the test substance. In accordance with the present invention, the promoter region of the *CaNik1* gene is operably linked to the luciferase gene. Gene activity is thus linked to luciferase activity, which can then be measured quantitatively, with a luminometer, as a bioluminescent reaction.

The present invention is described further below by reference to the following examples, which are illustrative only.

Example 1. PCR Amplification to Determine a *CaNik1* Probe

The following, deoxyinosine-containing, degenerate primers were designed that encompassed the highly conserved regions of the two component response regulators *LemA* (Hrabak & Willis, *J Bacteriol* 174: 3011-3020 (1992)), *BarA* (Nagasawa et al., *Escherichia coli. Mol Microbiol*, 6: 799-807 (1992)) and *SLN1* (Ota & Varshavsky, *Science* 262: 566-569 (1993)), respectively: 1) (SEQ ID NO:5) S1b1: 5-GAATTGAGAACGCCTITIAATGG-3, which corresponds to the histidine-autokinase domain; 2) (SEQ ID NO:6) S1b2: 5-AGICCTAAGCCA GTACCACC-3, which corresponds to the ATP-binding domain; and 3) (SEQ ID NO:7) S1b3: 5-TTAGGCATCTGGACITCCAT, which corresponds to the response regulator domain. S1b1 served as a 5'-end primer for PCR amplifications. The S1b1/S1b2 and S1b1/S1b3 pairs were used to amplify PCR products using the Hot-start wax gem (Perkin, Elmer) protocol. The Hot-start wax gem protocol which generates PCR products used the following reaction mixture: 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.2 mM MgCl<sub>2</sub>, 100 μM dNTP, 50 μM of each primer and 2.5 units of Taq polymerase, in a final volume of 100 μL. Conditions for PCR cycling included denaturation at 94°C for 1 min, annealing at 40°C for 1.5 min and extension at 72°C for 2.5 min. For all amplifications, *S. cerevisiae* genomic DNA was used as a control for the amplification of the two component hybrid kinase gene *SLN1*, to monitor the quality of the PCR products. PCR products were gel

purified and cloned into either PCR-Trap (Hunter Gen) or pGEM T-Easy (Promega Corp.). Three positive clones were chosen for each of the PCR products of the two sets of primer pairs.

pCN.5/3, pCN.5/11 and pCN.5/21 were chosen from the products of Slb1/Slb2; and pCN1.3/5, pCN1.3/13 and pCN 1.3/16 were chosen from the products of Slb1/Slb3.

#### Example 2. Isolation of CaNik1 Gene

To isolate a full-length gene, approximately  $8 \times 10^4$  plaques of a *C. albicans* genomic library were screened using a 1.2 kb DNA fragment isolated from pCN1.3/13, which spanned the histidine-autokinase (H1) and aspartyl receiver domain (D1). Lambda DNA from 20 positive clones was extracted and Southern blots probed with pCN1.3/13. Using combinations of primer pairs for the arms of the lambda DNA and either the degenerate primers for the histidine-autokinase domain (Slb1) or the response regulator domain (Slb3), lambda clones containing inserts larger than 4 kb were identified. The screen was performed with a high fidelity long PCR protocol (Boehringer Mannheim, Inc., Indianapolis, IN). Three lambda clones contained DNA fragments larger than 3 kb that flanked the upstream region of the histidine-autokinase domain and the downstream region of the aspartyl receiver domain. One of these clones, SA15.1, was chosen to determine the complete nucleotide sequence of the gene in both directions using the ABI automated sequencing system and fluorescent dideoxynucleotides as described earlier.

The DNA fragment generated by Slb1/Slb3 was used as a probe to screen a *C. albicans* EMBL3a lambda genomic library to identify clones containing the full-length gene. Of  $10^5$  pfu's, twenty positive clones were identified. Clone  $\lambda$ SA115.1, which contained a genomic fragment of approximately 4.8 kb with DNA flanking both the H1 and the D domains, was chosen for further characterization. The nucleotide sequence of the DNA insert was determined in both directions. The deduced amino acid sequence revealed an uninterrupted open reading frame of 1081 amino acids beginning with ATG as the initiation codon. The initiation codon was surrounded by an atypical Kozak consensus sequence CTCCAATGA, with cytosine at the -3 position (Kozak,

000270-15542460  
5 Nucleic Acids Res, 12: 857-871 (1984)). When total genomic DNA  
of *C. albicans* strain WO-1 was digested with a variety of  
restriction enzymes, and the resulting Southern blot hybridized  
under conditions of high stringency (65°C in Church-Gilbert  
10 hybridization buffer) (Church & Gilbert, *Proc Natl Acad. Sci*  
*USA* 81: 1991-1995 (1984)) with the 1.2 kb probe spanning the  
800 bp upstream of the gene, the banding pattern suggested that  
*CaNIK1* is encoded by a single copy gene. When total genomic  
DNA of strain WO-1 and strain 3153A was digested with *Bsa*AI or  
15 *Nci*I and hybridized with the 4.2 kb probe, the patterns were  
identical, but when *Tsp*I-digested DNA of the two strains were  
probed, the patterns differed, suggesting allelic differences  
exist between these strains. A comparison of the *CaNik1*  
sequence published recently by Nagahashi et al., *Candida*  
*albicans*. *Microbiology*, 144: 425-432 (1998) for strain IFO1060  
and the sequence we obtained for strain WO-1 in the present  
invention differ at seven nucleotide positions in the open  
reading frame of 3243 bp.

20 Example 3. Deletion of *CaNik1* in *C. albicans* Strain CA18

In order to generate a *CaNik1* deletion cassette, a DNA  
fragment of approximately 2.1 kb containing both the histidine-  
autokinase and aspartyl response regulator domains was  
amplified by PCR using as the template  $\lambda$ SA15.1 (Fig. 4a), which  
25 contained the 545 bp sequence upstream of the histidine-  
autokinase domain. The PCR fragment was gel-purified and  
cloned into the pGEM-T easy vector (Promega). The DNA insert  
was again excised from the recombinant plasmid with *Eco*RI and  
subcloned into a PUC18 vector (Life Technologies) at the *Eco*RI  
30 site. The resultant recombinant plasmid was designated  
pUNIK12.1 (Fig. 4b). A deletion construct pCNH35 was generated  
that spanned the histidine-autokinase and ATP binding-domains.  
To construct pCNH35, pUNIK12.1 plasmid DNA (Fig. 4b) was  
digested with *Afl*III and *Xho*I, and blunt-end repaired with the  
35 Klenow DNA polymerase I. The resultant plasmid DNA fragment  
was then gel purified and dephosphorylated with shrimp alkaline  
phosphatase (US Biochemical). A hisG-URA3-hisG cassette of 3.8  
kb from pMB9 was then ligated to derive the disruption cassette

(Fig. 4c). To isolate the *CaNik1* disruption cassette from pCNH35, plasmid DNA was digested with *Pst*I and the digested DNA extracted with phenol: chloroform. Approximately 25  $\mu$ g of the digestion mixture was used to transform strain CAI8, an *ade2<sup>-</sup>ura3<sup>-</sup>* derivative of wild type strain SC5314, by the lithium acetate protocol. Heterozygotes were selected for growth in minimal medium in the absence of uridine. Transformants were initially tested for the heterozygosity of one of the two *CaNik1* alleles by Southern blot hybridization of genomic DNA digested with *Pst*I. Positive heterozygotes were further confirmed by digesting genomic DNA with *Xho*I and by performing Southern blot hybridization. Because the genomic Southern revealed polymorphism between the two *CaNik1* alleles, two distinct heterozygotes, NNL6 (L stands for large allele) and NNS7 (S stands for small allele) were selected. The heterozygote NNS7 was chosen to generate the knock-out for the second copy of the *CaNIK1* gene. Prior to the knock-out of the second copy, NNS7 was subjected to the 5-FOA selection protocol to convert it from uridine prototrophy to auxotrophy. Loss of the *URA3* gene was again confirmed by digestion with *Xho*I and Southern blot analysis. In the final step, a single clone, NNS7.1.1, which was heterozygous for the L allele of the *CaNik1* locus and *URA3<sup>+</sup>*, was subjected to a second round of transformation with pCNH35, and selected for growth on defined minimal medium lacking uridine. Transformants which had lost the second copy of *CaNik1* were selected by Southern blot hybridization. One of the 125 transformants obtained with the pCNH35-based cassette, HH80, contained a homozygous deletion.

#### Example 4. *CaNik1* Transcription

To test whether transcription of *CaNik1* was regulated by high frequency phenotypic switching, Northern blots of polyA<sup>+</sup>mRNA of white and opaque phase cell growth cultures of strain WO-1 were probed with the DNA fragment spanning the H1 and ATP binding domains of *CaNik1*. The *CaNik1* transcript was detectable at very low levels in both white phase and opaque phase cells throughout the exponential phase of growth and in stationary phase. The level of *CaNik1* transcript per cell

remained constant throughout white phase cell growth, but increased steadily during opaque phase cell growth, reaching a level per cell roughly twice that of white phase cells at stationary phase (Fig. 5). Hypha-forming cells of both *C. albicans* strain WO-1 and *C. albicans* strain 3153A contained slightly higher levels of polyA<sup>+</sup> *CaNik1* transcript than budding cells. The hypha-to-bud ratio of polyA<sup>+</sup>-containing *CaNik1* transcript in strain WO-1 and strain 3153A was 1.2 and 1.3, respectively.

Example 5. Functional Characterization of the *CaNik1* Null Mutant of Strain CAI8

To test whether the *CaNik1* deletion mutant HH80 underwent switching, we first had to characterize switching in this strain using a low dose ultraviolet irradiation protocol that increases switching frequencies. Cells were treated with ultraviolet irradiation for 0, 5, 10, 20 and 40 sec, and the percent kill as well as the frequency and type of switch variants were assessed on modified Lee's medium. The proportions of CAI8 and HH80 cells killed after 5, 10, 20, and 40 sec were similar. Identical variant phenotypes were stimulated by UV in both CAI8 and the homozygous deletion strain HH80. However, the frequency of variants induced by comparable levels of UV-irradiation was consistently lower in strain HH80, and this was true in a repeat experiment. For instance, 20 sec of UV irradiation resulted in 10.6% and 2.6% variants in CAI8 and HH80 cells, respectively. These results demonstrate that the *CaNik1* gene product modulates phenotypic switching.

Since deletion of the *nik-1*<sup>+</sup> gene in *N. crassa* affects the morphology of hyphae, especially at high osmotic strength (Alex et al., *Proc Natl Acad Sci USA*, 93: 3416-3421 (1996)), the capability of the *CaNik1*- minus HH80 strain to form hyphae and the morphology of those hyphae were compared to that of the parent strain CAI8 and a URA3<sup>+</sup> isogenic strain CAI8U5 at 0, 1.0 and 1.5 M NaCl. Under the regime of pH-regulated dimorphism, CAI8, CAI8U5, and HH80 cells formed buds at pH 4.5 and hyphae at pH 6.7. The kinetics of evagination for the three strains

at low and high pH were similar at the three tested salt concentrations. At 1.5 M NaCl, the proportion of cells that formed evaginations at low and high pH was dramatically reduced in all three strains. The morphology of the hyphae that formed at pH 6.7 at 0, 1.0, and 1.5 M NaCl were comparable in the three strains. However, there was a significant and reproducible lag in hyphal growth at 1.5M NaCl in HH80 after 300 min. These results demonstrate that the *CaNik1* gene product is not essential for hypha formation under the regime of pH regulated dimorphism, but its presence enhances hypha formation at high ionic strength.

Finally, growth of the *CaNik1* deletion mutant HH80 was tested at 25°C and 37°C for differential sensitivity to osmotic strength and a variety of inhibitors. Patches of budding cells of CAI8, CAI8U5 and HH80 were plated on agar containing modified Lee's medium alone or with one of the following ingredients: 1.0 or 1.5M NaCl; 1M sorbitol; 0.8M KCl; 0.5M Mg<sub>2</sub>SO<sub>4</sub>; 20 or 40 µg per ml calcofluor; 1, 2 or 4 mg per ml caffeine; 10 or 20 mg per ml hygromycin; 0.002 or 0.004 µg per ml echinocandin; and 0.2 or 0.4M polymyxin B. In three independent experiments, no qualitative differences were observed between the growth of the control strains and the mutant strain HH80 for any of the tested conditions.

All publications and patent applications referred to in this specification are indicative of the level of skill of those in the art to which the invention pertains.

Other objects, features and advantages of the present invention will become apparent from the foregoing detailed description and examples. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given only by way of illustration.

Table 1

Conditions used to test the effect of gene deletion	*Phenotypic effect in HH80
1. Growth kinetics in	
a) Lee's modified broth	Similar to SC5314, CAI8U5, and CAI8
b) YPD broth	Similar to SC5314, CAI8U5, and CAI8 <sup>20</sup> .
2. Growth on agar plates with Lee's modified medium or YPD broth supplemented with:	
a) None	++++
b) 1M NaCl	++
c) 1.5M NaCl	+
d) 1M KCl	++
e) 1.2M Sorbitol	++++
f) 0.5m MgSO <sub>4</sub>	++
g) Caffeine (1-4 mg/mL)	v
h) Calcofluor (20-40 µg/mL)	+++ <sup>v</sup>
i) Echinocandin (0.002-0.004 µg/mL)	±
j) 2% Trehalose	++++
k) 2% Raffinose	++++
l) 1M Xylitol	++++
m) 10% Glycerol	++++
3. Switching	
a) spontaneous frequency	No effect
b) UV-stimulated frequency	Decreased
c) repertoire of switch phenotype	No effect
4. Hypha-induction under the regime of pH-regulated dimorphism.	
<u>with no osmotic shock:</u>	
a) time for 50% evagination	No effect
b) morphology of hypha	No effect
c) growth of hyphal filaments	
with osmotic shock using 1.5M NaCl	
i) time for 50% evaginations	decreased in both wild type and the mutant
ii) morphology of hyphae	no difference between wild hyphae and the mutant
iii) growth of hyphal filaments	the growth of the hyphae after 300 min was reduced in the mutant as compared to that in wild type

**Table 1 (cont'd)**

In order to assess the effect of gene deletion on growth, exponentially grown cells of wild type (SC5314), parental auxotrophic strain used to delete *NIK1* gene (CAI8), URA3<sup>+</sup> derivative of CAI8 (CAI8U5) and homozygous deletion mutant (HH80) were serially diluted and spot plated on agar plates with or without supplements in the medium. In all the growth medium used in this study, 2% glucose served as a carbon source except in the growth medium containing raffinose, trehalose and glycerol. The symbol "v" denote variable growth. Growth of the cultures were qualitatively assessed as very good (++++), good (+++), fair (++) , poor (+), poor to no growth ( $\pm$ ).  $\circ$  indicates that colonies were very small (less than 1mm) as assessed by the colony size on agar plates spread with cultures to generate 50 to 100 individual colonies. The growth of the cultures were assessed after 2 or 3 days incubation both at 25°C and 37°C.

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WHAT IS CLAIMED IS:

1. An isolated polynucleotide that codes for a protein that is involved in phenotypic switching in *Candida albicans* and that hybridizes, under stringent conditions, to polynucleotide sequence of SEQ ID NO:1.

2. A polynucleotide according to claim 1, having the sequence of SEQ ID NO:3.

3. A polynucleotide according to claim 1, wherein said protein displays kinase activity.

4. A method for screening compounds to identify pharmaceutical candidates for effectively inhibiting the pathogenicity of yeast, comprising the steps of (A) providing a plurality of cells from yeast species exhibiting phenotypic switching, at least some of which contain (i) a polynucleotide according to claim 1 and (ii) a promoter that is operably linked to said polynucleotide, such that said plurality produces said protein; then (B) bringing said plurality into contact with a test substance; and (C) assessing what effect, if any, said test substance has on the expression of said polynucleotide.

5. A method according to claim 4, wherein step (C) comprises monitoring the level of said protein produced by said plurality.

6. A method according to claim 4, wherein step (C) comprises monitoring the level of mRNA encoded by said DNA and produced by said plurality.

7. A method according to claim 4, wherein step (C) comprises monitoring the level of kinase activity within said plurality, wherein said kinase activity typifies said protein.

8. A method according to claim 4, wherein a promoter is operably linked to a reporter gene and wherein step (C) comprises monitoring the level of transcription of the reporter gene after contact between said plurality and said test substance.

ABSTRACT OF THE DISCLOSURE

5 A *Candida albicans* gene, *CaNik1*, is involved in phenotypic  
switching which is significant because of a direct correlation  
between the switching and the level of virulence of the  
organism. A method of screening for anti-fungal pharmaceutical  
10 candidates entails bringing a test substance into contact with  
cells containing a *CaNik1* gene or a variant thereof and then  
monitoring the effect, if any, on the level of expression of  
the gene.

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Sib1

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GAGATTAGAACACCATTGAATGGGATTATTGGWATGACYCAGTTGTCRCTTGATACAGAG 1590  
GluIleArgThrProLeuAsnGlyIleIleGlyMetThrGlnLeuSerLeuAspThrGlu 530

H1

TTGACRCAGTACCAACGAGAGATGTTGTCGATTGTGCATAACTTGGCAAATTCCTTGTTG 1650  
 LeuThrGlnTyrGlnArgGluMetLeuSerIleValHisAsnLeuAlaAsnSerLeuLeu 550

ACCATTATAGACGATATATTGGATATTTCTAAGATTGAGGCGAATAGAATGACGGTGGAA 1710  
 ThrIleIleAspAspIleLeuAspIleSerLysIleGluAlaAsnArgMetThrValGlu 570

CAGATTGATTTTTTCATTAAGAGGGACAGTGTTTGGTGCATTGAAAACGTTAGCCGTCAA 1770  
 GlnIleAspPheSerLeuArgGlyThrValPheGlyAlaLeuLysThrLeuAlaValLys 590

GCTATTGAAAAAACCTAGACTTGACCTATCAATGTGATTCATCGTTTCCAGATAATCTT 1830  
 AlaIleGluLysAsnLeuAspLeuThrTyrGlnCysAspSerSerPheProAspAsnLeu 610

ATTGGAGATAGTTTTAGATTACGACAAGTTATTCTTAACCTGGCTGGTAATGCTATTAAG 1890  
 IleGlyAspSerPheArgLeuArgGlnValIleLeuAsnLeuAlaGlyAsnAlaIleLys 630

N

TTTACTAAAGAGGGGAAAGTTAGTGTTAGTGTGAAAAAGTCTGATAAAATGGTGTTAGAT 1950  
 PheThrLysGluGlyLysValSerValSerValLysLysSerAspLysMetValLeuAsp 650

AGTAAGTTGTTGTTAGAGGTTTGTGTTAGCGACACGGGAATAGGTATAGAGAAAGACAAA 2010  
 SerLysLeuLeuLeuGluValCysValSerAspThrGlyIleGlyIleGluLysAspLys 670

G1

TTGGGATTGATTTTCGATACCTTCTGTCAAGCTGATGGTTCTACTACAAGAAAGTTTGGT 2070  
 LeuGlyLeuIlePheAspThrPheCysGlnAlaAspGlySerThrThrArgLysPheGly 690

Sib2

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GlyThrGlyLeuGlyLeuSerIleSerLysGlnLeuIleHisLeuMetGlyGlyGluIle 710

G2

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 SerAsnIleArgTyrThrArgGlnThrGluGlnLeuLeuPropheSerSerHisTyrVal 750

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 LeuPheValSerThrGluHisThrGlnGluGluLeuAspValLeuArgAspGlyIleIle 770

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 GluLeuGlyLeuIleProIleIleValArgAsnIleGluAspAlaThrLeuThrGluPro 790

GTGAAATATGATATAATTATGATTGATTGATAGAGATTGCCAAAAAGTTGAGGTTGTTA 2430  
 ValLysTyrAspIleIleMetIleAspSerIleGluIleAlaLysLysLeuArgLeuLeu 810

TCGGAGGTTAAATATATTCCGTTGGTTTTGGTCCATCATTCTATTCCACAGTTGAATATG 2490  
 SerGluValLysTyrIleProLeuValLeuValHisHisSerIleProGlnLeuAsnMet 830

AGAGTATGTATTGATTTGGGGATATCTTCCTATGCAAATACGCCATGTTTCGATCACGGAC 2550  
 ArgValCysIleAspLeuGlyIleSerSerTyrAlaAsnThrProCysSerIleThrAsp 850

TTGGCCAGTGCGATTATACCAGCGTTGGAGTCGAGATCTATATCACAGAACTCAGACGAG 2610  
 LeuAlaSerAlaIleIleProAlaLeuGluSerArgSerIleSerGlnAsnSerAspGlu 870

TCGGTGAGGTACAAAATATTACTAGCAGAGGACAACCTCGTCAATCAGAACTTGCAAGTT 2670  
 SerValArgTyrLysIleLeuLeuAlaGluAspAsnLeuValAsnGlnLysLeuAlaVal 890

Fig. 1a

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AGGATATTAGAAAAGCAAGGGCATCTGGTGGAAGTAGTTGAGAACGGACTCGAGGCGTAC 2730  
ArgIleLeuGluLysGlnGlyHisLeuValGluValValGluAsnGlyLeuGluAlaTyr 910

GAAGCGATTAAGAGGAATAAATATGATGTGGTGTTGATGGATGTGCAAATGCCT 2784  
GluAlaIleLysArgAsnLysTyrAspValValLeuMetAspValGlnMetPro 928  
D

Fig. 1b

**Fig. 2a**

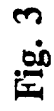
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 GluAlaLeuGlyGluMetAspAlaLeuLysThrLysIleAsnGlnMetValPheAsnLeu 480  
  
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 LysSerGluPheLeuAlaAsnMetSerHisGluIleArgThrProLeuAsnGlyIleIle 520  
  
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 IleValHisAsnLeuAlaAsnSerLeuLeuThrIleIleAspAspIleLeuAspIleSer 560  
  
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 GlnCysAspSerSerPheProAspAsnLeuIleGlyAspSerPheArgLeuArgGlnVal 620  
  
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 IleLeuAsnLeuAlaGlyAsnAlaIleLysPheThrLysGluGlyLysValSerValSer 640  
  
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 ValLysLysSerAspLysMetValLeuAspSerLysLeuLeuLeuGluValCysValSer 660  
  
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Fig. 2b

AATATTGAAGATGCAACATTGACTGAGCCGGTGAAATATGATATAATTATGATTGATTTCG 2400  
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 Arg\*\*\* 1082

Fig. 2c





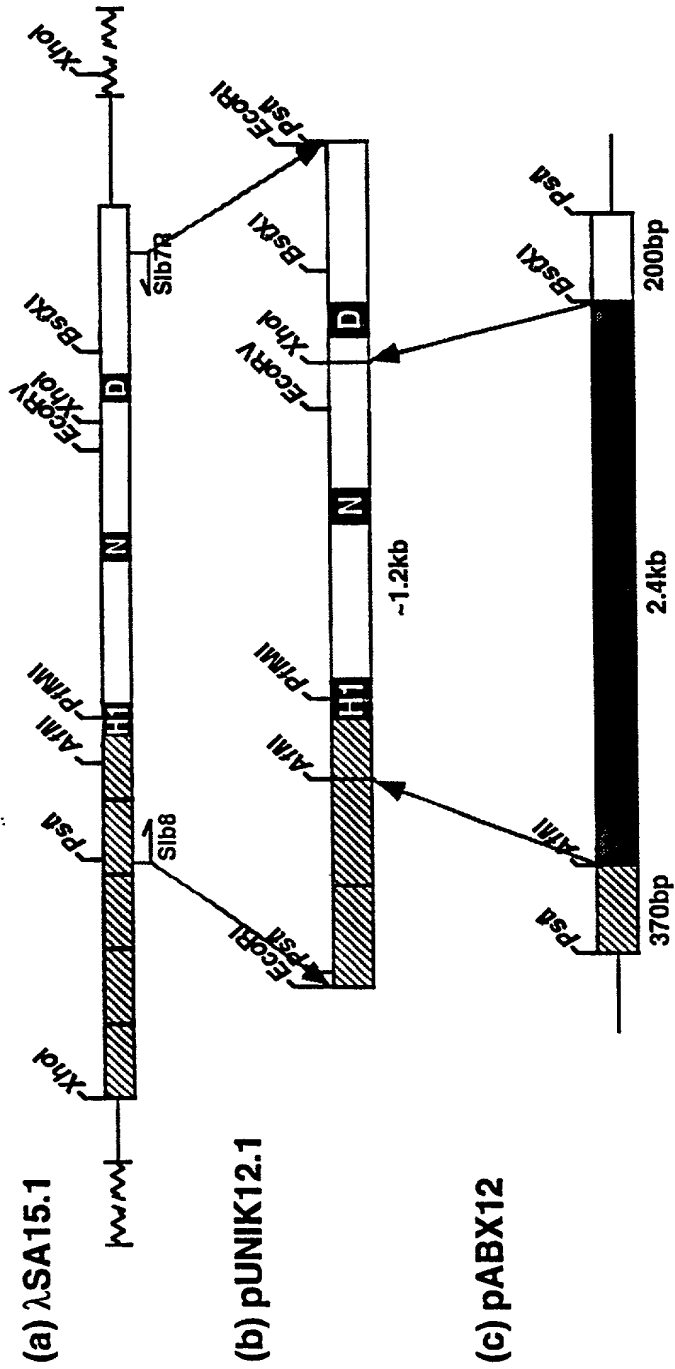


Fig. 4

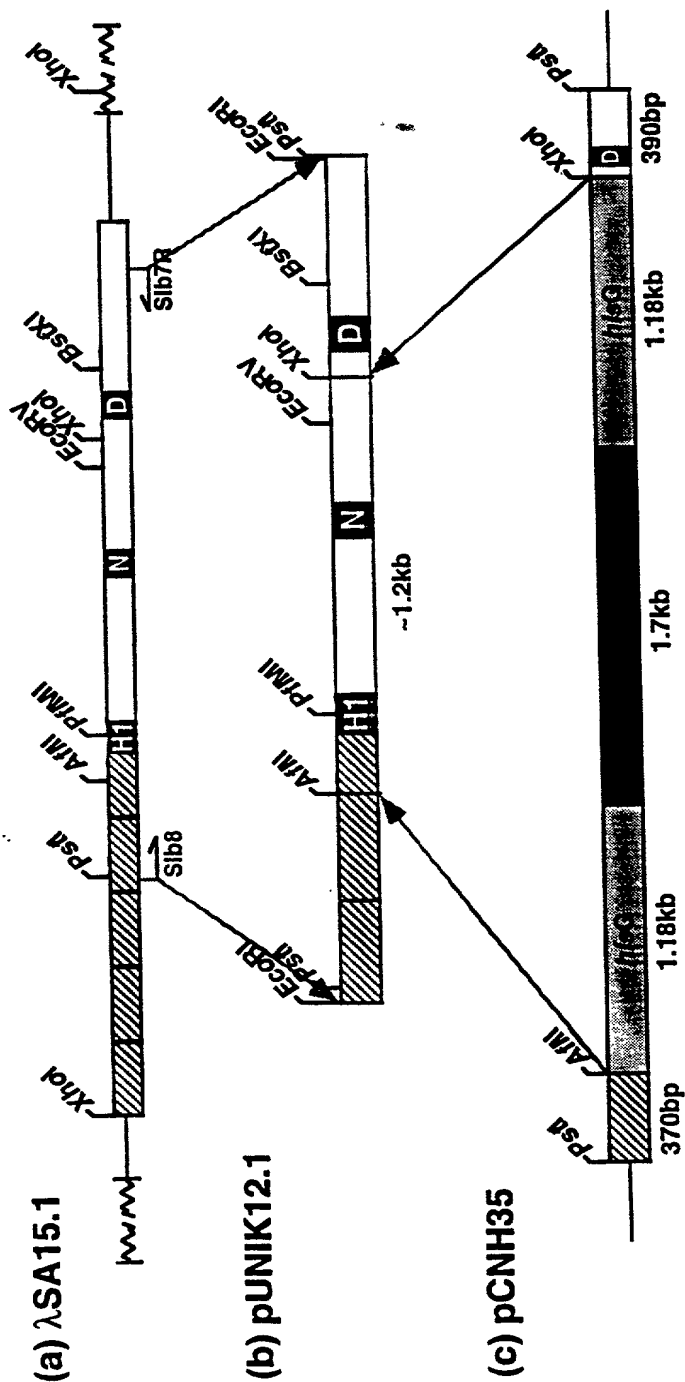


Fig. 5

**DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I HEREBY DECLARE:

THAT my residence, post office address, and citizenship are as stated below next to my name;

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

CANDIDA ALBICANS TWO-COMPONENT HYBRID KINASE GENE, CaNik-1, AND USE THEREOF

the specification of which (check one)

       is attached hereto.

  X   was filed on June 5, 1998 as United States Application Number or PCT International Application Number PCT/US98/11658 and was amended on                      (if applicable).

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

THAT I do not know and do not believe that the same invention was patented or described in any printed publication in any country, or in public use or on sale in the United States of America, for more than one year prior to the filing date of this United States application;

THAT I do not know and do not believe that the same invention was first patented or made the subject of an inventor's certificate that issued in any country foreign to the United States of America before the filing date of this United States application if the foreign application was filed by me (us), or by my (our) legal representatives or assigns, more than twelve months (six months for design patents) prior to the filing date of this United States application;

THAT I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment specifically referred to above;

THAT I believe that the above-identified specification contains a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention, and sets forth the best mode contemplated by me of carrying out the invention; and

THAT I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I HEREBY CLAIM foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number	Country	Foreign Filing Date	Priority Claimed?	Certified Copy Attached?

I HEREBY CLAIM the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

U.S. Provisional Application Number	Filing Date
60/048,914	06/JUNE/1997

I HEREBY CLAIM the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Application Number	Parent Filing Date	Parent Patent Number

I HEREBY APPOINT the following registered attorneys and agents of the law firm of FOLEY & LARDNER to have full power to prosecute this application and any continuations, divisions, reissues, and reexaminations thereof, to receive the patent, and to transact all business in the United States Patent and Trademark Office connected therewith:

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and I request that all correspondence be directed to:

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I UNDERSTAND AND AGREE THAT the foregoing attorneys and agents appointed by me to prosecute this application do not personally represent me or my legal interests, but instead represent the interests of the legal owner(s) of the invention described in this application.

I FURTHER DECLARE THAT all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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